Accelerated Wound Repair in ADAM-9 Knockout Animals

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ADAM-9 belongs to a family of transmembrane, disintegrin-containing metalloproteinases (ADAMs) involved in protein ectodomain shedding and cell–cell and cell–matrix interactions. Although the functions of many ADAM family members are known, the specific biological function of ADAM-9 is still unclear. In this study, we have analyzed ADAM-9 temporal and spatial distribution during wound healing. We showed increased ADAM-9 transcript expression during the first 7 days post-wounding and, by immunolocalization, detected ADAM-9 in all migrating and proliferating keratinocytes from days 3 to 7. In older 14-day-old wounds, ADAM-9 expression was restored. We have investigated the role of this protein in the healing process following excisional wounding. Animals deficient in ADAM-9 showed accelerated wound repair compared with control littermates. No alterations in neutrophil, leukocyte, and macrophage infiltration were observed. However, re-epithelialization was significantly faster in Adam-9 −/− than control wounds. Although no differences in proliferation were observed *in vivo* and *in vitro*, increased migration of keratinocytes was responsible for this effect. These results show the previously unreported role of ADAM-9 in wound repair by regulating keratinocyte migration through modulation of collagen XVII shedding.

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INTRODUCTION

The process of wound healing begins the moment the tissue is injured. After the formation of a clot that seals the wound, inflammation, re-epithelialization, granulation tissue formation, and matrix remodeling are required to complete healing by restoring the structure and function of the tissue. Proteolysis is required in many of these stages and is directed both to the degradation of damaged tissue and remodeling of the new extracellular matrix as well as to the release and activation of growth factors and cytokines generating signals, which directly or indirectly promote cell growth and migration (Bergers and Coussens, 2000; Blobel, 2002). Different families of proteinases (serine, cysteine, aspartic proteinases, and metalloproteinases) have been implicated in wound healing during which they have an important role in cell migration and extracellular matrix remodeling. In addition to these enzyme families, ADAM (A Disintegrin And Metalloproteinase) proteases, initially described as sheddases of cell membrane proteins, have also been shown to be involved in the degradation of extracellular matrix proteins (Millichip *et al.*, 1998; Alfandari *et al.*, 2001; Schwettmann and Tschesche, 2001; Martin *et al.*, 2002). ADAM-9, -10, and -17 are expressed more in the epidermis and less in the dermal compartment, and are involved in the cleavage of collagen XVII as well as of cell surface receptors, such as E-cadherin, both thereby modulating cell migration (Franzke *et al.*, 2002; Maretzky *et al.*, 2005). In addition, ADAM-9 cleaves and releases epidermal growth factor (EGF), fibroblast growth factor receptor 2 IIIb as well as heparin-binding EGF-like growth factor from cells (Peduto *et al.*, 2005), all of which have pivotal functions in wound healing.

Previous studies have shown that activated CD4 + T lymphocytes and lymphoma cells express ADAM-9 and -17 *in vitro* (Wolf *et al.*, 2003). In addition, expression of ADAM-9 has been detected in circulating monocytes as well as in macrophages and shown to be involved in the formation of multinuclear giant cells in both bone and skin (Namba *et al.*, 2001; Mackiewicz *et al.*, 2005; Ma *et al.*, 2006), thereby implying the putative involvement of ADAM-9 in inflammatory processes.

Increased expression of ADAM-9 was observed *in vivo* during vessel repair following angioplasty (Ward *et al.*, 2001) as well as during horse skin repair (Lefebvre-Lavoie *et al.*, 2005). In a recent study, we detected ADAM-9 protein and transcripts in both the epidermal and dermal compartments of normal human skin (Zigrino *et al.*, 2007). Furthermore, we have also shown that ADAM-9 interacts with integrin-β1 on keratinocytes whose interaction with the adhesive domains of ADAM-9 leads to the secretion of matrix metalloproteinase (MMP)-9 and modulation of cell migration (Zigrino *et al.*, 2007). However, it is still unclear as to the role of ADAM-9 in skin and during physiological processes such as wound healing *in vivo*.

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Abbreviations: ADAM, a disintegrin and metalloproteinase; con-A, concanavalin-A; FAD, mixture of Ham's F12 and Dulbecco's modified Eagle's medium; MMP, matrix metalloproteinase

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In this investigation, using mice carrying a complete inactivation of the Adam-9 gene, we have addressed the role of ADAM-9 in wound healing processes *in vivo*. Further, we showed that wound re-epithelialization *in vivo* is modulated by the expression and activity of ADAM-9 in keratinocytes. Finally, we have shown that the absence of this protease reduced cleavage of collagen XVII and resulted in increased migration of keratinocytes *in vivo* and *in vitro*.

RESULTS

ADAM-9 expression during normal wound healing and in human chronic wounds

In a recent study, we could show that ADAM-9 protein and transcripts are detected in both the epidermal and dermal compartments. To test whether ADAM-9 expression is modulated during wound healing, we have generated full-thickness excisional wounds (4 mm Ø) on the backs of C57/Bl6 control animals. At the indicated time points, the mice were killed and the wounds processed for histology and transcript analysis. High ADAM-9 transcript levels were detected between 6 hours and 6 days post-wounding compared with unwounded skin and with wounds 7–14 days after injury (Figure 1a).

ADAM-9 protein was expressed in epidermal keratinocytes in unwounded skin. On wounding, its expression was associated with all migrating and proliferating keratinocytes from days 3–11 (Figure 1b). On day 14, wound expression of ADAM-9 was found in all keratinocytes with prominent expression in suprabasal layers comparable to the expression pattern observed in non-wounded skin.

To analyze the role of ADAM-9 during wound repair, we have used ADAM-9 knockout (KO) and wild-type (WT) animals. Despite Adam-9 deficiency, mice developed normally, were fertile, showed no gross phenotypic abnormalities, and were indistinguishable from their WT littermates and controls, being in agreement with published data (Weskamp *et al.*, 2002).

Histological examination of the back skin of 8-week-old mice revealed no obvious difference between Adam-9 WT and KO animals in overall skin morphology (Figure 2h and e).

Likewise, expression analysis of keratin 14, a cytokeratin protein expressed in the basal keratinocyte layer of the skin, and loricrin, a protein representative for the *stratum granulosum*, indicated normal keratinocyte differentiation in Adam-9 KO mice (Figure 2).

Cutaneous wound healing in Adam-9 KO mice

To study the physiological role of Adam-9 during wound healing, we generated full-thickness excisional wounds on the back skin of 8-week-old Adam-9 KO and WT littermate

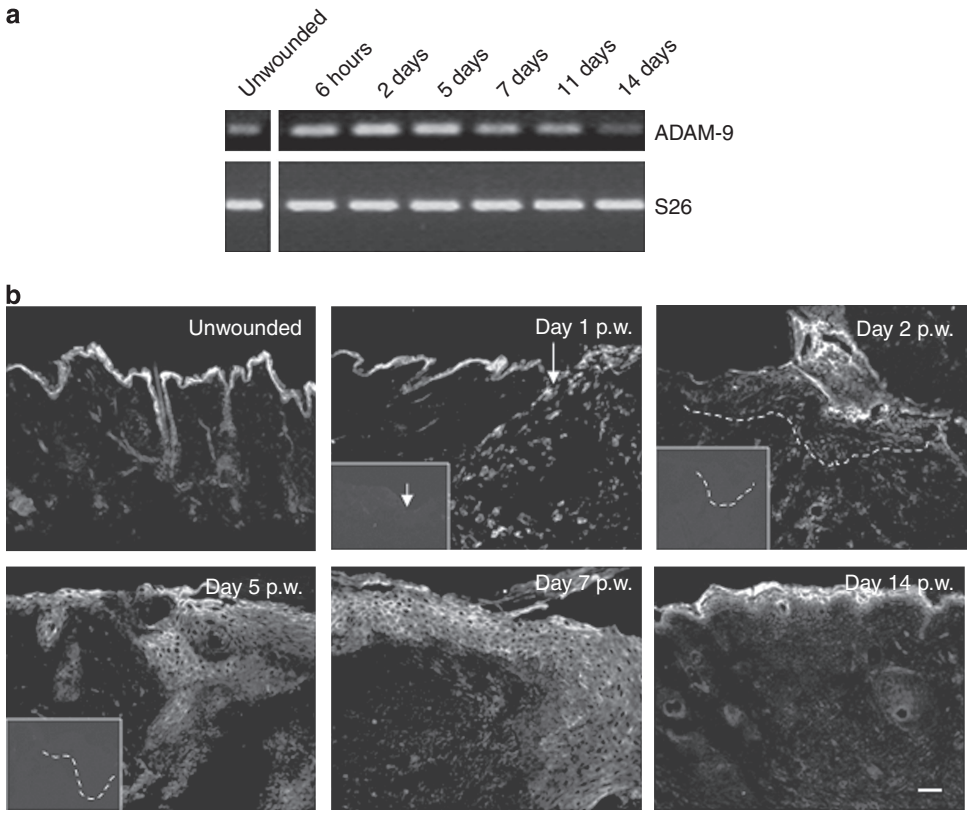

Figure 1. ADAM-9 expression during wound healing and in chronic wounds. (**a**) Reverse transcription-PCR of ADAM-9 on RNA isolated from wounds of control animals at different time points. Amplification of S26 was used as control. (**b**) Shows an overview of the wounds stained for ADAM-9 localization at days 1, 2, 5, 7, and 14 after wounding as well as an unwounded control. The small insert represents the stained Adam-9 knockout (KO) wounds. The arrow marks the wound margin and the broken line underlines the epithelial tongue. Scale bar, 100 μm.

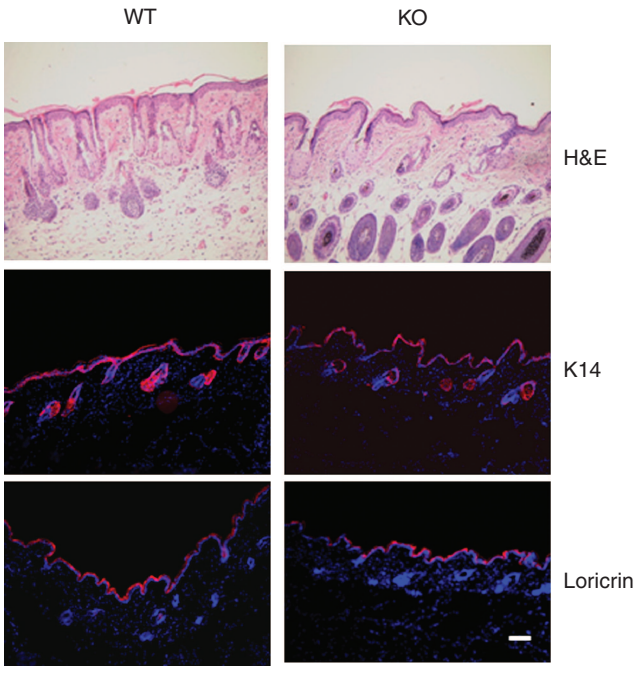

Figure 2. Analyses of epidermal tissue morphology and differentiation in ADAM-9 knockout (KO) animals. Overview of the skin stained for hematoxylin/eosin (H&E). Keratin 14 (K14) and Loricrin were used to analyze epidermal differentiation and early and late keratinocyte differentiation, respectively, in wild-type (WT) and Adam-9 −/− (KO) animals. Scale bar, 100 µm.

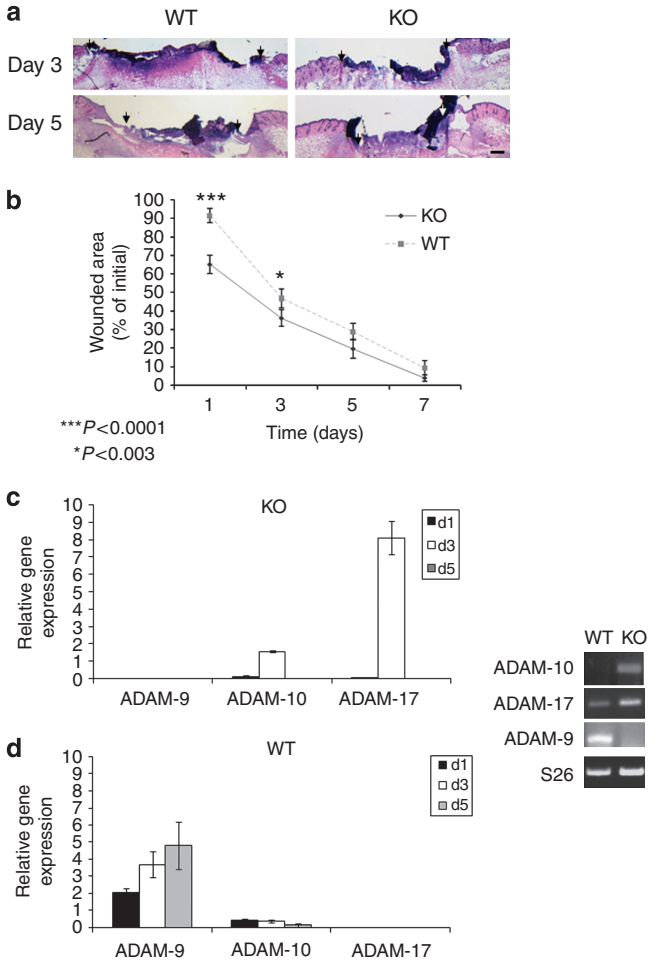

Figure 3. Accelerated wound closure in knockout (KO) mice. The healing process of 4 mm full-thickness excisional wounds in the back skin of *Adam-9* mutant (KO) and control mice (wild-type, WT) were followed over 7 days ($n=16$ per genotype and time point). (**a**) Photomicrographs and histological sections indicated accelerated wound healing in *Adam-9 KO* mice. Arrowheads indicate wound edges. Scale bar, 200 µm. (**b**) Closure of wounds from *Adam-9 KO* and WT mice at days 2, 5, and 7 ($n=4$ wounds per genotype and time point) was judged microscopically and the number of open wounds was calculated in percent. (**c**) ADAM-9, -10, and -17 were amplified by quantitative PCR on RNA isolated from four different animals (graphs) at days 1,3, and 5 post-wounding. On the right, reverse transcription-PCR of these transcripts was, in addition, performed on pooled RNA wounds isolated from four different WT and KO animals. Amplification of S26 was used as control.

mice. On injury, wound closure was assessed by histological analysis of wounds (Figure 3a) and by following the wound closure kinetics that was expressed as wound area percentage versus initial area. Measuring the area of the wounds as well as the distance between the leading wound edges on sections of Adam-9 KO and WT mice at different time points after wounding revealed a significant acceleration in wound closure as early as day 1 post-wounding ($P<0.0001$; Figure 3b).

These data show that re-epithelialization after cutaneous injury is affected by the loss of Adam-9 expression/function, suggesting that ADAM-9 is required during the initial phases of wound healing but can be functionally replaced later on by other ADAMs. Furthermore, an upregulation of ADAM-10 and -17 transcripts at day 3 post-wounding (Figure 3c) was detected by quantitative and semi-quantitative PCR (Figure 3c, right) and would suggest that these ADAMs may replace ADAM-9 function to lead to normal skin repair.

Granulation tissue formation at the wound sites

Expression of ADAM-9 in tissues is quite broad and dermal fibroblasts as well as cells of the immune system express ADAM-9, activated lymphocytes, peripheral blood mononuclear cells and macrophages (Namba *et al.*, 2001; Mackiewicz *et al.*, 2005; Ma *et al.*, 2006); however, apart from the documented activity in cell fusion for the formation of giant cells, it is unclear whether ADAM-9 expression is also necessary for efficient inflammatory reaction. To analyze the inflammatory response to wounding tissue sections from days 1–5, wounds were analyzed for the infiltration of macrophages and neutrophils. As shown in Figure 4, infiltration of neutrophils and macrophages (positive for CD68 and F4/80) was comparable in wounds of both Adam-9 KO and WT mice.

Keratinocyte re-epithelialization of wounds

ADAM-9 expression and activity in keratinocytes have been shown to modulate cell migration by one or more of the following processes: either cleaving collagen XVII, inducing expression of MMP-9 in migrating keratinocytes

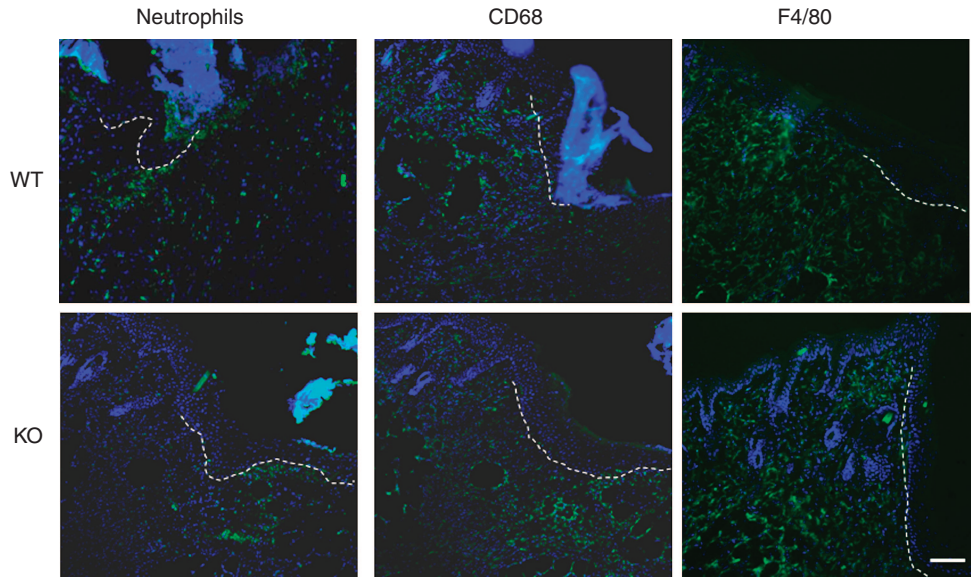

Figure 4. Histological analyses of the granulation tissue at wound sites. Localization of macrophages using anti-CD68, as well as F4/80 and neutrophils using anti-neutrophil antibodies (both in green), showed no obvious differences in the infiltration of these cells in the granulation tissue of both knockout (KO) and wild-type (WT) animals. Day 5 wounds are shown here. Scale bar, 100 μm.

(Zigrino *et al.*, 2007), and/or modulating the shedding of heparin-binding EGF-like growth factor (Izumi *et al.*, 1998). To show a possible function of ADAM-9 in keratinocyte re-epithelialization *in vivo*, we performed histological examination of the wounds at early time points. This analysis revealed significantly increased length of keratinocyte migratory tongue over the wound bed of KO compared with WT animals ($P<0.001$). These data were obtained by measuring the length of the migrated tip (marked by the red dotted line) of the wound at days 3 and 5 post-wounding (Figure 5a).

The observed increase in length of the migrating tip was not due to increased proliferation as no differences in the number of Ki67 positive cells, used as proliferation markers, were observed at the edge (arrow) or into the migrating tip of both wounds (Figure 5b). These data indicate that the loss of ADAM-9 leads to increased keratinocyte re-epithelialization of the wounds.

We had previously shown that the interaction of keratinocytes with the adhesive domains of ADAM-9 leads to the secretion of MMP-9 and modulation of keratinocyte migration (Zigrino *et al.*, 2007). MMP-9 expression in wounds at days 3 and 5 shows a slight reduction in the epithelial tongue of migrating keratinocytes in Adam-9 KO but not WT animals (data not shown). However, a difference in the total MMP-9 activity in wound lysates was not observed by gelatin zymography (data not shown), but the possibility remains that the difference is below the level of detection. Furthermore, it is unclear at this point whether MMP-9 *in vivo* derives from the epithelial cells or the stromal cell.

Analysis of collagen XVII distribution in wounds from WT and KO animals shows an increased basolateral immunopositive reaction in the keratinocytes of the migrating tip of the KO, which was not obvious in WT animals (Figure 6a, white arrows). This is in agreement with previous studies showing that shedding of collagen XVII results in decreased keratinocyte motility (Franzke *et al.*, 2002). Furthermore, analysis of tissue lysates from wounds at days 1 and 5 shows the presence of shed collagen XVII at day 1 post-wounding only in lysates from WT wounds but not from KO-extracted wounds (Figure 6b).

In vitro analysis of isolated epidermal keratinocytes

To analyze whether the altered migration of keratinocytes *in vivo* results from the alteration of a molecular path in these cells or in their communication with stromal cells, we have isolated primary keratinocytes and studied their migratory potential *in vitro* by time-lapse video microscopy. After reaching confluency, keratinocytes were treated with mitomycin to block proliferation. ''Wounds'' were made in the center of the confluent monolayer of mitotically arrested cells and cell migration was monitored over 24 hours. Quantification of migrated distance in KO and WT keratinocytes shows increased migration 6 hours post-wounding that was most significant after 12 hours. This effect is promptly visible in the phase contrast images shown in Figure 7.

These data show that ADAM-9 ablation in keratinocytes results in increased cell migration resulting from a keratinocyte-dependent effect.

In addition, analysis of supernatants from cultured primary keratinocytes shows constitutive shed collagen XVII released in the supernatants of WT but not KO keratinocytes at time 0, which progressively increased after 6 and 24 hours (Figure 8a). Shed collagen XVII was detected in supernatants of KO keratinocytes only after 24 hours. Furthermore, zymographic analyses of these supernatants show a normal secretion of MMP-9 and -2 (Figure 8b).

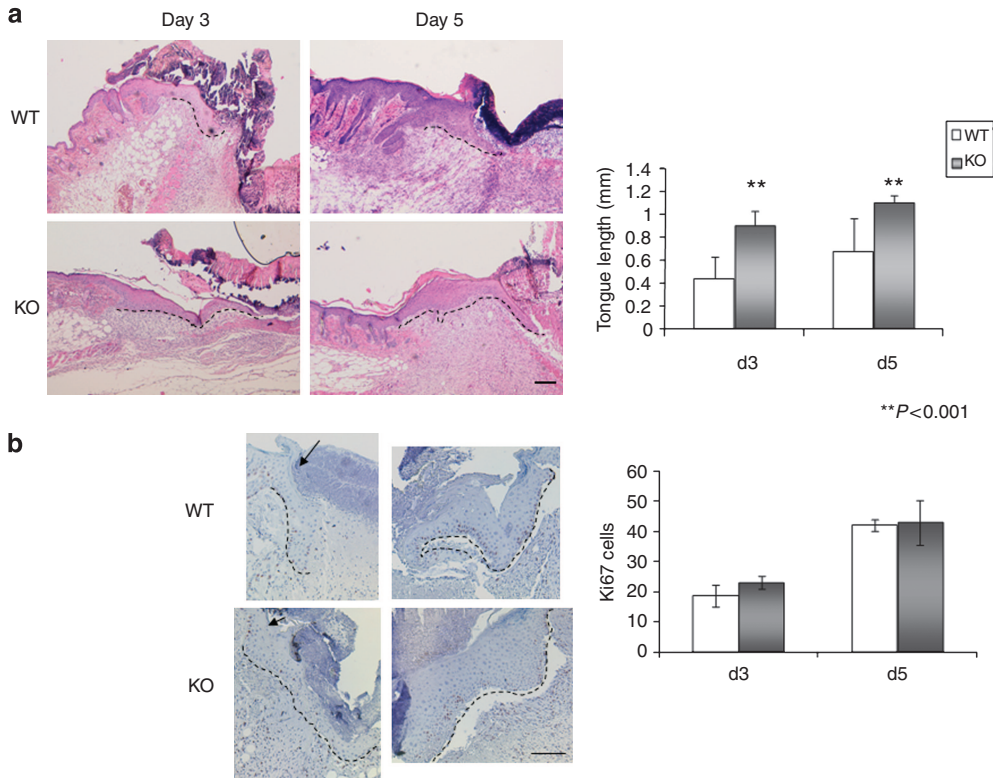

Figure 5. Increased length of migrating epithelial tongue. (**a**) Epithelia migrating tongues, marked by broken line, of knockout (KO) and wild-type (WT) wounds stained for hematoxylin/eosin. The length of the tongues was measured in four different wounds from each WT and KO animal at days 3 and 5 post-wounding with the result shown graphically. (**b**) Sections of 3- and 5-day-old wounds were stained with antibodies for Ki67 to examine keratinocyte proliferation. The number of positive cells was counted from the beginning and through the tongue in four different wounds of WT and KO animals and the results are shown on the right. Scale bar, 100 μm.

Transient transfection of human soluble ADAM-9 (active or catalytically inactivated by the mutation E>A) in KO mouse keratinocytes led to the presence of the metalloproteinase in the cell culture supernatant (Figure 8c) and the associated collagen XVII shedding. The inactive form of ADAM-9 did not show these effects. Furthermore, rescue of ADAM-9 KO keratinocytes by the addition of recombinant ADAM-9 was shown to reduce migration by 20 and 30% after 4 and 8 hours respectively, thus attenuating the accelerated ''migration phenotype'' observed in the KO keratinocytes (Figure 8d).

Thus, these results indicate collagen XVII shedding as the main molecular process altered in Adam-9 KO, which is associated with increased keratinocyte migration.

DISCUSSION

The specific tissue functions of ADAM proteases in skin are well understood. These molecules have different domains that display different functions, such as membrane fusion, cytokine and growth factor shedding, and cell adhesion and migration (Edwards *et al.*, 2008). The sheddase activity of epidermal ADAMs may have an important role in keratinocyte biology; for instance, the shedding of heparin-binding EGF-like growth factor and other EGFR ligands has been shown to regulate keratinocyte proliferation and migration (Izumi *et al.*, 1998).

Among the ADAMs, ADAM-9, -10, and -17 are expressed by keratinocytes in intact human epidermis. In addition, ADAM-9 is also produced by fibroblasts (Zigrino *et al.*, 2007). In murine skin as well as in human skin, ADAM-9 has a preferential localization throughout the epidermal layer (Zigrino *et al.*, 2007). Interestingly, we observed a significant induction of ADAM-9 transcripts in human chronic wounds, thus suggesting a role for this protein in pathological skin repair. Chronic wounds with delayed, insufficient, or missing wound closure are characterized by pathologic inflammation, fibroblast senescence, and uncontrolled proteolysis (Diegelmann and Evans, 2004). Excess proteolysis may lead to destruction of wound extracellular matrix, disturbance of cell migration, or degradation of momentous growth factors and their receptors. However to understand what is the function of ADAM-9 in this context, we needed to understand which function it may have during physiological wounding. To address this question, we have analyzed the distribution of this protease in normal wound healing.

During wound healing, expression of this protein was observed in all migrating and proliferating layers of the epidermis with an increased level detected in the initial phases post-wounding (Figure 1). During these initial phases within a few hours after injury, epidermal keratinocytes at the wound edge detach from the underlying basement membrane

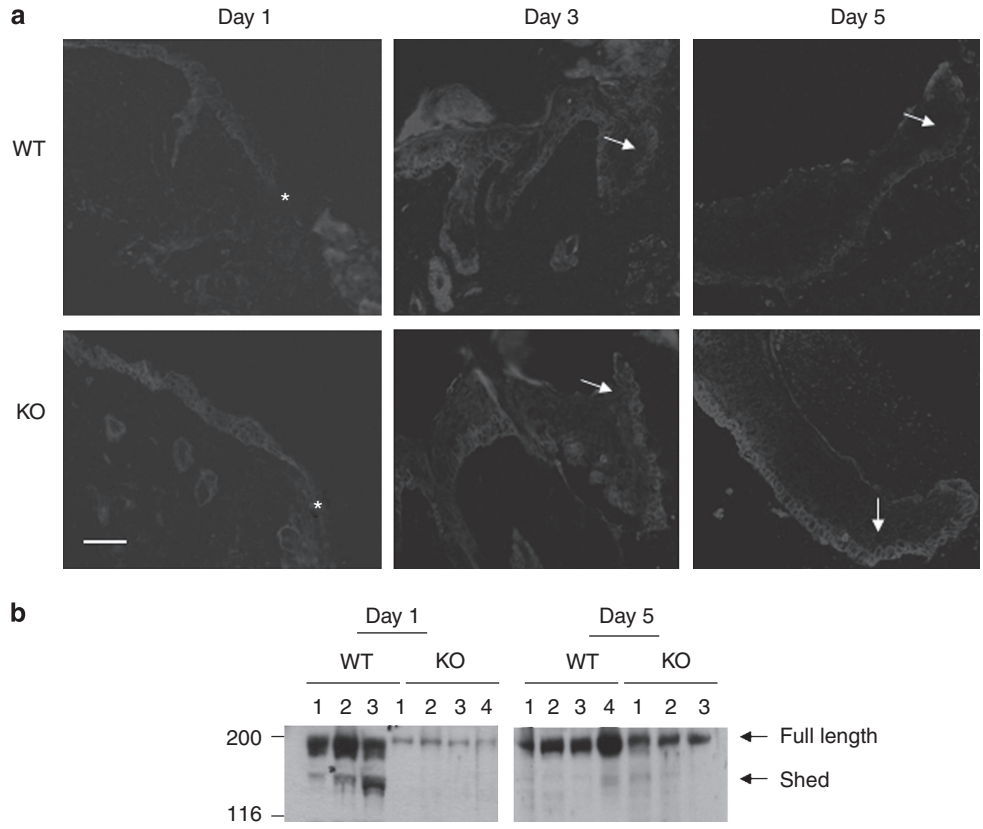

Figure 6. Collagen XVII expression in Adam-9 knockout (KO) and wild-type (WT) wounds. (**a**) Collagen XVII was immunolocalized in wounds at days 1, 3, and 5 post-wounding using specific antibodies to the NC15 domain of the protein. White arrows indicate areas of the tip where expression of collagen XVII is detected, and the stars at day 1, the edge of the wound. Note that in the epithelial migrating tip of WT wounds at day 5, staining for collagen XVII is almost absent, whereas it is still present in the keratinocytes of KO tips. Scale bar, 100 μm. (**b**) Collagen XVII was analyzed by immunoblot in wound extracts (5 μg) from different wounds/animals (numbered from 1 to 4) at days 1 and 5 post-wounding. The shed and full-length forms of the protein are indicated on the right.

and from adjacent cells and start to migrate into the wound, typically underneath the scab (Martin, 1997). After 2–3 days, wound keratinocytes distant from the wound edge also obtain a hyperproliferative phenotype, and thus increased the cell number and migration to fill the gap in the epithelium (Martin, 1997).

In wound healing experiments performed with animals with complete ADAM-9 ablation, we could observe an accelerated wound re-epithelialization. Thus, taking together the expression profile and the altered re-epithelialization, we could suggest that ADAM-9 may be involved in regulating either migration or cell proliferation necessary for re-epithelialization. Inflammatory processes that occur in the early phases of wound healing (Martin, 1997) seemed to be unaffected as we could not see any differences in neutrophil and macrophage infiltration. Furthermore, we have not detected differences in cell proliferation in wounds from days 1, 3, and 5, but have detected more extended epithelial migrating tongues in days 3 and 5 wounds from KO animals compared with WT animals. These data suggest that accelerated re-epithelialization in ADAM-9 KO animals may result from increased epithelial migration. Indeed, this seems to be the case as *in vitro* analysis of keratinocytes isolated from KO animals shows that these cells migrate faster than do the littermate controls.

Few data are available on the putative function of ADAM-9 during epidermal migration in wound healing and these come from *in vitro* experiments. For instance, shedding of collagen XVII from keratinocytes by ADAMs -9, -10, and -17 has been implicated in regulating motility of the cells because of the inhibitory effect of the shed collagen XVII ectodomain on migration (Franzke *et al.*, 2002, 2009). In addition, ADAM-9 has been shown to bind α3β1-integrin on human keratinocytes lines and induce the expression of MMP-9 and subsequent cell migration in a manner that involves activation of ERK-mitogen-activated protein kinase (Zigrino *et al.*, 2007). Thus, the lack of ADAM-9 may result in altered migration of keratinocytes because of impairments in MMP-9 expression and/or alteration of collagen XVII shedding. In support of this, studies with MMP-9-deficient mice showed enhanced re-epithelialization of cornea and skin, probably because of the enhanced epithelial cell proliferation (Mohan *et al.*, 2002). However, we could not detect differences in MMP-9 activity in lysates from wounds from days 1 to 5 and also in supernatants of primary keratinocytes, but only a slight difference in MMP-9 expression in the epithelial migrating

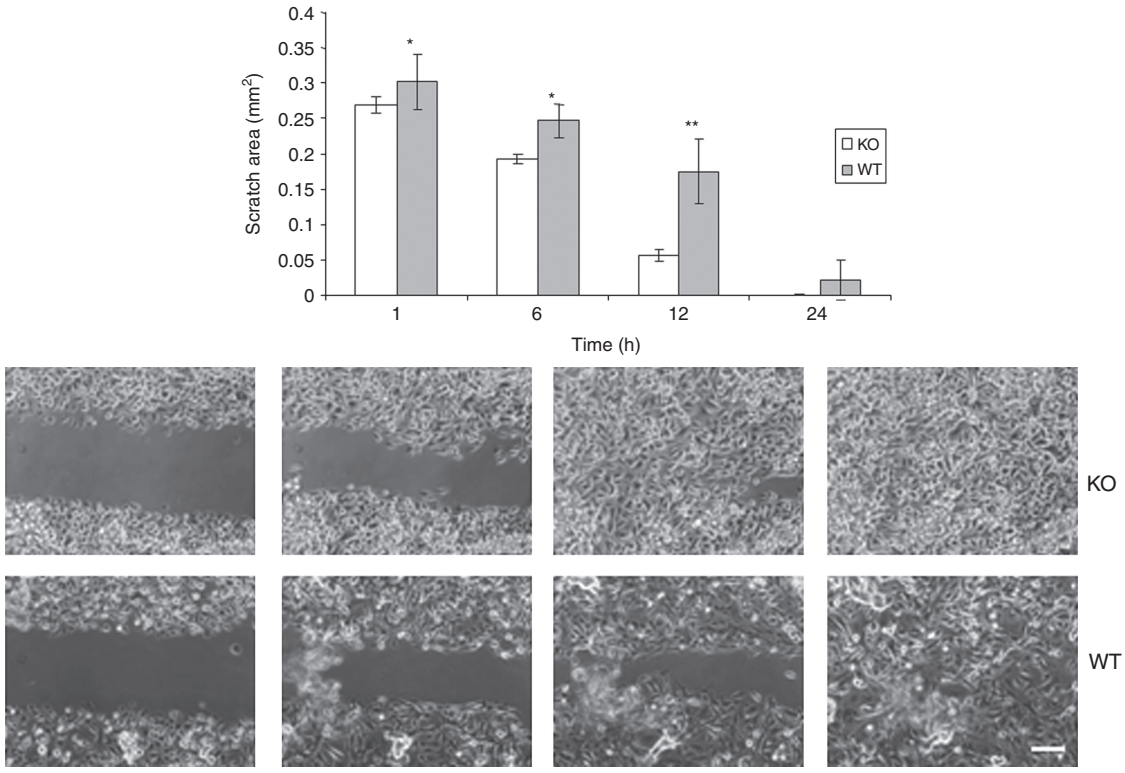

Figure 7. Migration of Adam-9 knockout (KO) primary keratinocytes is enhanced. Time-lapse video microscopy of primary keratinocytes isolated from wild-type (WT) or Adam-9 KO animals seeded onto collagen type I. After wounding the monolayer, cell migration was monitored over 24 hours, and the area covered by the cell sheet at the indicated time points was calculated. Area is denoted as mm^2 and is representative of two experiments performed in sextuple. Representative microphotographs from the migration experiment are shown. Scale bar, 100 μm. $*P<0.03$; $**P<0.003$.

keratinocyte tip in KO animals day 5 post-wounding. These results suggest that it is the MMP-9 localization more than the alterations in secretion that contribute to the observed wound-healing phenotype. Future experiments will be aimed at specifically addressing this issue.

Furthermore, Leivo *et al.* (2000) have shown that during re-epithelialization, collagen XVII is expressed in the keratinocytes distal to the wound edge, but not in the leading cells of the epithelial tip. Shedding of collagen XVII may lead to changes in the biological activities of the molecules, by altering their functions in ligand binding, cell attachment, or signal transduction. It has recently been shown by *in vitro* re-epithelialization assays that induced shedding of collagen XVII results in decreased keratinocyte motility (Franzke *et al.*, 2002). Therefore, the shed ectodomain is likely to have a role in cell–matrix signaling through interaction with cell surface receptors, leading to changes in lateral migration of keratinocytes. Indeed, we could observe a persistent expression of collagen XVII in the migrating epithelial tip at days 3 and 5 post-wounding in KO but not WT animals, thus suggesting that the expression of this molecule is not altered events but shedding events. In agreement with this, we could detect shed forms in wound extracts from day 1 wounds from WT but not KO animals (data not shown). However, we failed to detect any further shedded form in the later time points (data not shown). Thus, the detection *in situ* of the collagen XVII we observed in day 3 wounds from WT may be the result of detection of shed collagen XVII, which is likely to be further cleared in day 5 wounds where the expression is significantly reduced. In support of this hypothesis, *in vitro*, primary keratinocytes isolated from Adam-9-depleted animals showed impaired constitutive collagen XVII shedding compared with cells isolated from WT animals. Furthermore, transfection of murine keratinocytes from null animals with proteolytically active ADAM-9 restored collagen XVII shedding, whereas proteolytically inactivated ADAM-9 mutant (E>A) did not.

In addition, replacement of active ADAM-9 in KO keratinocytes partly rescued the accelerated migration of keratinocytes *in vitro*. Lack of a complete rescue may derive from the use of a soluble active protein, which we have used to circumvent the low efficiency of transfection of primary murine keratinocytes. Thus, expression of a membrane-bound form of ADAM-9 might have directly docked in the vicinity of the transmembrane substrate and more efficiently shed it completely restoring the cell phenotype.

It is possible that increased expression of ADAM-10 and -17 in day 3 KO wounds replaces additional unknown functions of ADAM-9 that minimize the effects of ADAM-9 ablation for skin repair. However, even if an increase in ADAM-10 expression in KO wounds at day 3 could be detected, our data would nevertheless suggest that ADAM-9 may be

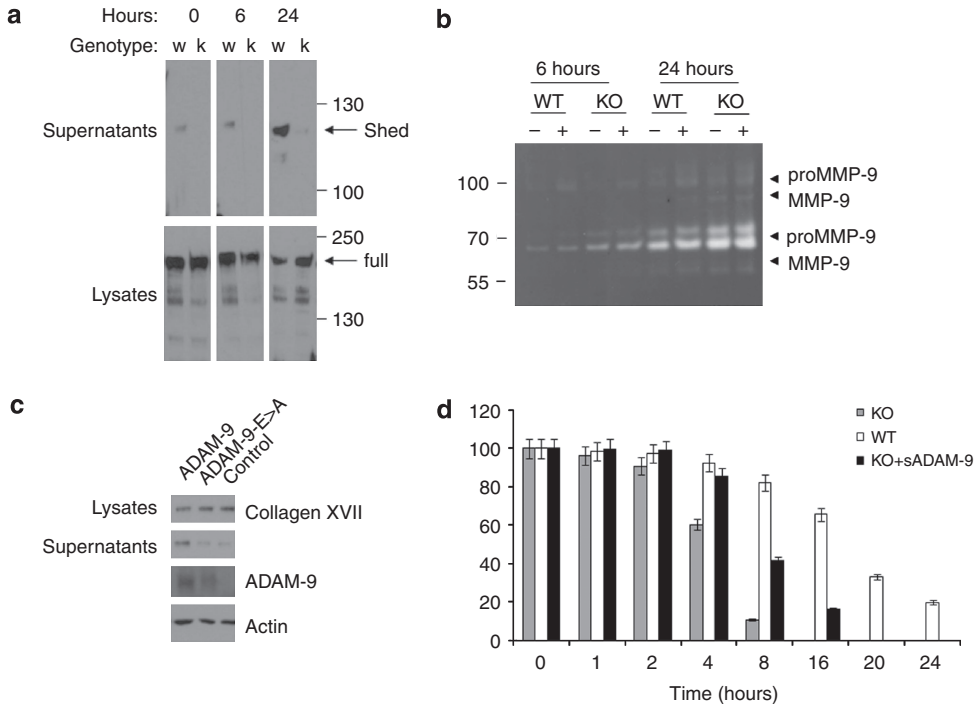

Figure 8. Reduced constitutive shedding of collagen XVII but no altered matrix metalloproteinase (MMP)-9 secretion is detected in Adam-9 knockout (KO) primary keratinocytes. (**a**) Decreased collagen XVII shedding was detected by immunoblot in supernatants of murine keratinocytes from KO compared with Adam-9 wild-type (WT) mice *in vitro*. Cells (lysates) and media (supernatants) were sampled after 0, 6, and 24 hours and immunoblotted with anti-NC15 antibodies. (**b**) MMP-9 secretion was analyzed by gelatine zymography in supernatants of phorbol myristate acetate-treated (50 mμ) primary KO and WT keratinocytes collected at the indicated time points. (**c**) Collagen XVII shedding was restored by transient transfection (24 hours) of murine KO keratinocytes with the active but not inactivated (E>A) human ADAM-9. ADAM-9 immunoblot confirmed expression of the proteins and actin was used as loading control. (**d**) Time-lapse video microscopy of primary keratinocytes isolated from WT or Adam-9 KO animals seeded onto collagen type I. After wounding the monolayer, KO keratinocytes were incubated in the presence of 300 ng recombinant soluble ADAM-9 and cell migration was monitored over 24 hours, and the area covered by the cell sheet at the indicated time points was expressed as a percentage of the initial area.

primarily responsible for the initial shedding events occurring in keratinocytes during the first phases of wound repair.

In conclusion, it is possible that increased wound repair in ADAM-9 KO animals results from the lack of induced collagen XVII shedding, which occurs during the activation of the wound edge keratinocytes immediately after wounding and, in addition, from alterations in MMP-9 localization *in vivo* in migrating keratinocytes.

These results show that increased expression of ADAM-9 during wound repair results in negative regulation of re-epithelialization. Further studies will be aimed at investigating the role of ADAM-9 in chronic ulcers to open up new ways for therapeutic intervention.

MATERIALS AND METHODS

Antibodies

For immunodetection analysis, goat polyclonal antibodies raised against murine ADAM-9 were purchased from R&D Systems (Wiesbaden, Germany). Ki67 antibodies were from Dako, Hamburg, Germany (1:100) and rabbit anti-loricrin (1:100) and rabbit anti-keratin 14 (1:250) from Babco, Richmond, CA, and rat anti-CD68 and rat anti-neutrophils from Serotec, Düsseldorf, Germany (1:100). Collagen XVII antibodies were a kind gift from Prof L. Brückner-Tudermann (University of Freiburg, Germany). Rabbit anti-MMP-9 antibodies were from Chemicon, Hofheim, Germany (1:100). Actin was detected using a mouse mAb (MP Biomedicals, Irvine, CA). Purified control IgG was purchased from Dako.

Cells, cell culture, and transfection

Murine epidermal keratinocytes were isolated from newborn skin as previously described (Sudbeck *et al.*, 1997). Keratinocytes were cultured on collagen-coated dishes in mixture of Ham's F12 and Dulbecco's modified Eagle's medium (FAD) medium containing 50 μM Ca^{2+} in the absence or presence of mitomycin C-treated 3T3 fibroblasts (4 μg ml^{-1}) as feeders. The FAD medium (DMEM/Ham's F12, 3:1; Invitrogen, Karlsruhe, Germany) contained 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 10% fetal calf serum, 5 μg ml^{-1} insulin, 1 ng ml^{-1} EGF, 10^{-10} mol l^{-1} cholera toxin, and 24 ng ml^{-1} adenine. Primary keratinocytes were seeded on collagen type I coated (30 μg ml^{-1}) 12-well plates at 8% confluency and transfected with 2 μg of DNA per well with the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. After 5 hours, the medium was replaced and 50 μg ml^{-1} ascorbic acid was added to allow for hydroxylation of collagen and proper triple helix formation. Fresh FAD medium without serum supplemented with 50 μg ml^{-1} ascorbic acid was

added the next day after transfection and incubated for a further 24 hours. Proteins released in the supernatants and lysates were prepared as later described.

The plasmid encoding the soluble ADAM-9 was a kind gift from Alex Toker (Beth Israel Deaconess Medical Center, Harvard Medical School). The transformer site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) was used to introduce a specific mutation of the ''catalytic'' glutamic acid in the metalloproteinase active site (Glu → Ala; E>A) shown to result in loss of proteolytic activity of ADAM-9 toward insulin β-chain (Roghani *et al.*, 1999). Insertion of mutation was confirmed by DNA sequencing.

Wound healing experiments

C57/Bl6 (8–10 weeks old) mice or C57/Bl6-SV129 Adam-9 −/− (KO) and littermate controls (WT) were anesthetized with a single intraperitoneal injection of ketamine/xylazine. These animals were kindly provided by Prof C. Blobel (Hospital for Special Surgery, New York) (Weskamp *et al.*, 2002). The hair on the back was shaved and the skin wiped with 70% ethanol. Two full-thickness excisional wounds (4 mm diameter) were created on the back of each animal by excising the skin and *panniculus carnosus* as previously described (Werner *et al.*, 1994). The wounds were allowed to dry to form a scab. Animals were killed at different time points after wounding and the complete wounds, including the epithelial margins, were isolated. Wounds were bisected in caudocranial direction and the tissue embedded in O.C.T. Compound (Tissue Tek, Vogel, Giessen, Germany), and used for immunohistochemistry. Histological analysis was performed on serial sections from the central portion of the wound. Cryosections (5 μm) of the wounds were stained with hematoxylin and eosin (Shandon, Frankfurt, Germany), documented, and measured using a Leica (DMLB, Wetzlar, Germany) microscope. Alternatively, wounds were excised, collected in phosphate-buffered saline (PBS) containing1% SDS, and homogenized using a Mixer mill (3 minutes, 30 Hz; Retsch, Germany). Extracted proteins in the supernatants from centrifuged extracts were transferred to new tubes and stored at −80 °C till use. All animal experiments were performed in compliance with the German Law for Welfare of Laboratory Animals and approved by the Regierungspräsidium Köln, Germany.

RNA isolation and reverse transcription-PCR

Total DNase-treated RNA from the skin wounds was prepared using the RNAeasy Fibrous tissue Kit following the manufacturer's instructions (Qiagen, Hilden, Germany). Reverse transcription-PCR was performed according to the manufacturer's instructions (REDTaq ReadyMix PCR Reaction Mix, Sigma, Taufkirchen, Germany). Briefly, 1 μg RNA was reverse transcribed using oligodT as primer in a total volume of 25 μl. A volume of 2 μl of the cDNA was used to amplify specific transcripts by PCR. The following primers were used for amplification of murine ADAM-9: 5′-TCTGACCATCCCAACG TACA and 5′-GCTGTTGTGCAGAGGTTCAA; murine MMP-9: 5′-AGTTTGGTGTCGCGGAGCAC and 5′-TACATGAGCGCTTCCGG CAC (Kim *et al.*, 2000); murine ADAM-10 5′-GCAACATCTGGGG ACAAACT-3′, 5′-TTGCATATCCCTTCCTTTGC-3′; murine ADAM-12 5′-ATGCCAGAACATGGACATCA-3′, 5′-TGGGAAGGTCTGGGTA CTTG-3′; murine ADAM-15 5′-TGATGGACCCTGTTGTCAAA-3′, 5′-CAACAGAGGCTGGCTCCTAC-3′; and murine ADAM-17 5′-TT GAGCGATTTTGGGATTTC-3′, 5′-CATCCTCTGGTGGTCCAGTT-3′. Amplification of murine S26 was used for normalization: 5′-AATG TGCAGCCCATTCGCTG and 5′-CTTCCGTCCTTACAAAACGG (NM013765). PCR reactions were performed on 1 μl cDNA for 35 cycles (within the linear range of amplification): denaturation (94 °C, 1 minute), annealing (60 °C, 1 minute), and extension (72 °C, 1 minute). The products were then analyzed on 2% agarose gels in Tris/Borate/EDTA buffer.

For quantitative real-time PCR analysis, RNA was reversed transcribed into cDNA by using random hexamers and Superscript II Plus RNase H^- Reverse Transcriptase (Invitrogen, Carlsbad, CA). The level of expression was quantified by real-time PCR using the ABI RealTime 7300. One microliter of sample cDNA was added to the master mix and the amplification was performed in a total volume of 20 μl for 40 cycles. The thermal cycling conditions were set to 50 °C for 2 minutes and 95 °C for 10 minutes, followed by 40 cycles of amplification at 95 °C for 15 seconds and 60 °C for 1 minute for each cycle. Primers used for the amplification were previously described (Franzke *et al.*, 2009).

The relative expression levels were determined by normalizing expression to S26. Expression level of normalized samples is displayed in relative expression units, which were calculated by the comparative $\Delta\Delta C_t$ method according to the manufacturer's instructions.

Zymographic analysis

Equal amounts of proteins from serum-free conditioned media and wound extracts (see above) were analyzed by gelatin zymography as previously described (Zigrino *et al.*, 2001). Briefly, proteins were fractionated on 10% SDS–PAGE containing 1 mg ml^{-1} gelatin (bovine, Sigma, Deisenhofen, Germany). After electrophoresis, the gels were washed in 2.5% Triton-X 100 for 30 minutes before overnight incubation in metalloproteinase substrate buffer (50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$). Thereafter, the gels were stained with Coomassie Blue R 250 and the bands corresponding to gelatinase activities appeared white against the blue background.

Western blot analysis

Keratinocyte lysates were prepared by washing the cells twice in PBS followed by scraping them off on ice in RIPA buffer containing the protease inhibitors aprotinin (10 μg ml^{-1}), Pefabloc (0.25 mg ml^{-1}), and leupeptin (1 μg ml^{-1}). After overnight incubation at 4 °C, lysates were clarified by centrifugation at 16,000 × *g* and 4 °C for 20 minutes and the supernatant collected and stored at −20 °C till use. Protein concentration was determined using a commercial assay (MicroBCA, Perbio Science, Bonn, Germany). To analyze released collagen XVII, equal amounts of supernatants were incubated overnight with concanavalin-A-Separose (Amersham Pharmacia Biotech, Freiburg, Germany). After centrifugation at 1,000 *g* for 10 minutes, con-A-bound proteins were washed twice with PBS and bound proteins eluted in sample buffer containing 0.7 M β-mercaptoethanol. Eluted protein was further analyzed by western blotting. Wound lysates for this analysis were prepared as described above (''zymographic analysis''). For western blotting, proteins were fractionated by SDS–PAGE on 7% polyacrylamide gels under reducing conditions and transferred onto Hybond-C Super (Amersham Pharmacia Biotech). After blockage of nonspecific binding sites with 5% skimmed milk (w/v) in PBS containing 0.5% Tween (v/v), for analysis of phosphorylated proteins 5 mM sodium fluoride

was included, and the blots were incubated with the primary antibodies overnight at 4 °C. Bound primary antibodies were detected using a horseradish peroxidase-conjugated secondary antibody (1:2,000; Dako) and visualized with the ECL system (Amersham Pharmacia Biotech).

Cell migration assays

Cell migration assays were performed in 24-well tissue culture plates. Confluent layers of primary keratinocytes were treated with mitomycin-C (1.6 μg ml^{-1}) for two hours to arrest cell growth, then washed, and a scratch was produced in the center of the well. After washing twice with PBS and introducing fresh media, plates were placed on a microscope stage heated to 32 °C in a humidified atmosphere. For rescue experiments, keratinocytes were incubated in the presence of 300 ng recombinant soluble ADAM-9 (R&D Systems). Areas covered by cells at different time points were calculated using Cell^R software (Olympus Biosystems, Munich, Germany).

Immunolocalization

Cryosections (5 μm) of wounds were fixed in methanol (−20 °C) for 5 minutes (neutrophils, CD68), in acetone (−20 °C) for 5 minutes (keratin-14, loricrin), or in paraformaldehyde 4% for 5 minutes (Florin *et al.*, 2006). Cryosections were then blocked with 10% BSA (Sigma) in PBS containing 0.05% Tween and incubated with the primary antibodies diluted in the blocking solution overnight at 4 °C. The first antibodies are described above in the ''Antibodies'' paragraph. After three washes each of 5 minutes, bound primary antibodies were detected using goat anti-rabbit-Alexa 594 or rabbit anti-mouse-Alexa 488 (all diluted in PBS with BSA/Tween, Invitrogen, Karlsruhe, Germany) for 1 hour at room temperature. Nuclei were counterstained with 1 μg ml^{-1} 4,6-diamidino-2-phenylindole (Roche, Mannheim, Germany). Negative controls were performed using nonspecific IgG as primary antibodies.

For Ki67 immunodetection, cryosections were fixed in 100% methanol (4 °C) for 5 minutes and acetone (4 °C) for 10 minutes. After rinsing with PBS, endogenous peroxidase was quenched with 3% H_2O_2 in methanol for 10 minutes at room temperature. The cryosections were then blocked with 1% BSA (Sigma) in PBS, incubated with the primary antibodies diluted in blocking solution for 1 hour at room temperature, and followed by incubation with a peroxidase-coupled secondary antibody plus streptavidin-peroxidase complex and DAB staining (Vectastain ABC kit, Vector Laboratories, Burlingame, UK). Sections were counterstained with hematoxylin.

Statistics

Two-tailed Student's *t*-test was used for data analysis, with $P<0.05$ considered to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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